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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

**Determination of Optimal UV Stress Period before
Harvest for Maximizing Phytochemical Production of
Kale Cultivated in Plant Factories**

식물공장에서 재배되는 케일의 이차대사산물 최대
생산을 위한 수확 전 UV 스트레스의 최적 처리 시기
결정

BY

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AUGUST, 2017

**MAJOR IN HORTICULTURAL SCIENCE AND BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY**

**Determination of Optimal UV Stress Period before Harvest
for Maximizing Phytochemical Production of Kale Cultivated
in Plant Factories**

UNDER THE DIRECTION OF DR. JUNG EEK SON
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF
SEOUL NATIONAL UNIVERSITY

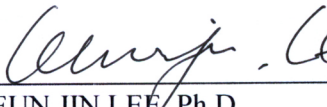
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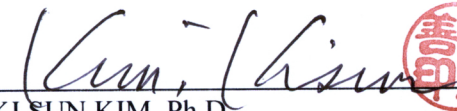
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Determination of Optimal UV stress Period before Harvest for Maximizing Phytochemical Production of Kale Cultivated in Plant Factories

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ABSTRACT

Recently ultraviolet-B (UV-B) has been used to improve the secondary metabolites of kales grown in plant factories. However, it is still unclear whether UV-B stress will be effective in enhancing the secondary metabolites at harvest because most of UV-B stresses were applied at early growth stage. The objectives of this study were to analyze the secondary metabolites accumulation at uneven UV-B distributions in leaves of matured kales at harvest stage using spatial chlorophyll fluorescence images and to determine the optimal UV stress period before harvest for maximizing secondary metabolites production of kales cultivated in plant factories. Kales (*Brassica oleracea* L. cv ‘Manchoo Collad’) were grown at a temperature of 20°C, photosynthetic photon flux density of 350 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and photoperiod of 16 h/8 h (light/dark) in a plant factory, and harvested at 42 days after transplanting. Light-emitting diodes (LED) with red:blue:white = 8:1:1 were used. At first, spatial chlorophyll fluorescence (F_v/F_m) of the plants at different UV-B intensities in leaves at harvest were measured by a leaf fluorescence image analyzer. And then, the plants were additionally exposed to 4.2 $\text{W}\cdot\text{m}^{-2}$ UV-B for 4 h a day from 5, 4, 3, 2, and 1

days before harvest (T5, T4, T3, T2, and T1, respectively). Fresh and dry weight, total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacity were compared at harvest. In the same leaves, F_v/F_m values were different according to locations exposed to different UV-B intensities, while no significances were observed in TFC and TPC. This indicated that accumulation of secondary metabolites is not always proportional to the local F_v/F_m values. UV-B stress decreased fresh and dry weights and increased secondary metabolites. TFC, TPC, and antioxidant capacity at T5 were significantly higher than any other treatments but dry weight was the lowest. Considering total amounts of secondary metabolites, T2 was the optimum treatment period, in which the concentration was lower but the dry weight higher than T5. This is because the dry weight had a greater effect on the total amount than the secondary metabolites concentration induced by UV-B radiation. To maximize the secondary metabolites of kales with UV-B treatment, it would be more efficient to use short-term stresses to minimize the loss of fresh and dry weights.

Additional key words: antioxidant capacity, chlorophyll fluorescence image, total flavonoid content, total phenolic content

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INTRODUCTION

Kale is a leafy green vegetable that belongs to the brassica family. Recently, kale have gained increased attentions due to their high contents of secondary metabolites (Olsen et al., 2009). Commercially, kale is processed into juice form to conveniently ingest the secondary metabolites. For stable supply of fresh kale, controlled plant production systems rather than open fields or greenhouses are required.

Plant factory can control the growth as well as the contents of secondary metabolites regardless of season by controlling indoor environments. Since cultivation in plant factories is onerous because of high initial investment and cost of maintenance (Lee et al., 2014), optimization of production quantity was tried by finding suitable growth conditions in plant factories (Fujiwara et al., 2011; Nagatoshi et al., 2015). In addition to the growth conditions, it is also important to find the harvest time to determine the cultivation period in plant factories (Okamura et al., 2014a; Okamura et al., 2014b). Kim (2016) reported that the optimum harvest time for kale for obtaining the maximum total secondary metabolites in plant factories.

To overcome economical limitations of plant factory, improvement of the quality of plants is one of the important issues. Recent researches have focused on promotion of secondary metabolite contents in plants by using environmental stresses. Several researches were conducted by using abiotic stresses such as ultraviolet (UV) radiation, high temperature, or drought enhance the contents of secondary metabolites (Oh et al., 2008; Tsormpatzidis et al., 2010; Brechner et al.,

2011; Lee et al., 2014; Lee et al., 2015). In particularly, Tilbrook et al. (2013) revealed that UV-B stress is effective to promote secondary metabolism process. Heijde and Ulm (2012) explained the relationship between UV-B radiation and a gene expression related with secondary metabolites.

From practical points of view, it is convenient to apply UV-B radiation to promote secondary metabolites in plant factory systems. Although UV-B stress has a disadvantage of suppressing plant growth, the loss of fresh weight can be minimized by applying the stress near the harvest time (Kim, 2016). Furthermore, because of uneven UV-B distributions on leaves in matured kale at harvest, the effect on UV-B radiation may be different. However, there are few studies to improve secondary metabolites by applying the stresses at harvest stage. The objective of this study was to analyze the relationship between spatial distribution of UV-B intensity and contents of secondary metabolites and to determine UV-B treatment period before harvest for maximization of the total secondary metabolites production.

LITERATURE REVIEW

Determination of optimal harvest time to maximize phytochemicals

Several researches were conducted to determine optimal harvest time to maximize phytochemicals in open fields (Rohloff et al., 2005; Pandino et al., 2013; Kim et al., 2016; Bilandzija et al., 2017). Similar methodologies were suggested for plant factories having controlled environments (Kozai, 2013). For vaccine-producing transgenic lettuces in plant factories, the cultivation period was determined by comparing the cultivation period and total yields per plant (Okamura et al., 2014b). Kim (2016) determined the optimum harvest time in plant factories to maximize the secondary metabolite production of kales.

Enhancement of secondary metabolites by abiotic stresses

Accumulation of secondary metabolites in plants is affected by the environments (Bartwal et al., 2013; Zandalinas et al., 2017). When the strong stresses, such as UV-B, are given, the contents of secondary metabolite in plants are enhanced (Harbaum-Piayda et al., 2010; Hejide and Ulm, 2012; Allorent et al., 2016). The contents of hypericin were increased by adding UV-B to St' John Wort (Brechner et al., 2011). Flavonoids also were promoted at UV-B stress by a well-known mechanism of action for UV-B (Tsormpatsidis et al., 2010). However, most of the UV-B treatment were relatively applied at early growth stage in the previous studies,

and there were few researches on UV-B application at the late stage near harvest (Lee et al., 2014).

Chlorophyll fluorescence image analysis

Chlorophyll fluorescence image analysis were used for non-destructive, spatial evaluations of the stressed degrees in plants (Lichtenthaler and Mische, 1997). This technique also provided the stress gradients over the whole leaf area (Lichtenthaler and Babani, 2000) Chlorophyll fluorescence image was useful to investigate the stress distributions according to position in plants under UV-B stress (Allorent et al., 2016). The analysis of fluorescence image provided useful tools to discriminate stressed plant based on a high sensitivity of PSII to stress (Buschmann and Lichtenthaler, 1998; Murchie and Lawson 2013). Autofluorescence in the blue and green regions is an interesting phenomenon, which is emitted by phenolic compounds with a wide range of roles in plant defense (Granum et al., 2015).

MATERIALS & METHODS

Plant material and growth conditions

Kales (*Brassica oleracea* L. cv ‘Manchoo Collad’, Asia Seed Company, Seoul, Korea) were cultivated in a plant factory module of Seoul National University. The seedlings were grown in deep flow culture under florescence lamps at a photosynthetic photon flux density (PPFD) of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$. Growing after three weeks, seedlings were transplanted in deep flow systems containing nutrient solutions developed by National Institute of Horticultural and Herbal Science (Choi et al., 2005) for *Brassica* with an EC of 1.2dS m^{-1} . The plants were grown at a temperature of 20°C , relative humidity of 70%, CO_2 concentration of $500 \mu\text{mol} \cdot \text{mol}^{-1}$, PPFD of $350 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, and photoperiod of 16 h/8 h (light/dark) in a plant factory, and harvested at 42 days after transplanting. Light-emitting diodes (LED) with red:blue:white = 8:1:1 and) were used as light sources.

UV treatments

The plants grown under the LEDs were additionally exposed to UV-B lamps ($4.2 \text{ W} \cdot \text{m}^{-2}$; Sankyo Ultraviolet Co. Ltd., Kanagawa, Japan) for 4 h a day. In Experiment 1, spatial chlorophyll fluorescence (F_v/F_m), total phenolic content (TPC), and total phenolic content (TFC) in leaves were analyzed at 38, 40, and 42 days after transplanting under different UV-B intensities (Table 1). In Experiment 2, fresh and dry weights, F_v/F_m , TPC, TFC, and antioxidant capacity (DPPH radical scavenging)

of the plants were measured and analyzed at different UV-B stress treatments (Table 2).

Leaf chlorophyll fluorescence

Chlorophyll fluorescence was measured in the third leaves from top that has exposed to the UV-B lamps. Chlorophyll fluorescence were measured at 4 h intervals by using a chlorophyll fluorescence meter (Handy PEA fluorimeter, Hansatech, Kings Lynn, UK). Kale leaves were adapted dark condition in a leaf clip for 30 min prior to the measurement. Measurement of F_0 (pulse intensity as above, duration of 100 ms) and F_m was obtained by saturated light pulse of $800 \text{ mmol m}^{-2} \text{ s}^{-1}$. The maximum PSII quantum yield (F_v/F_m) was calculated using the formula:

$$(F_m - F_0) / F_m.$$

Chlorophyll fluorescence image analysis

Chlorophyll fluorescence image was analyzed by using a chlorophyll fluorescence image analyzer (Open FluorCam FC 800-0, PSI, Brno, Czech Republic). Measuring light flashes (10 ms) for modulated chlorophyll fluorescence excitation were generated by a pair of red LED panels ($\lambda_{\text{max}} \sim 618 \text{ nm}$), and saturating light pulse (1 s, $2000 \text{ } \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) and actinic light by a pair of blue LED panels ($\lambda_{\text{max}} \sim 455 \text{ nm}$). Chlorophyll fluorescence kinetics were captured by a charge-coupled device camera with 12 bits and 96 pixels per inch resolution.

Sample preparation

Whole plants were stored at - 80°C overnight and then lyophilized for 120 h. Following freeze-dried, kale was crushed used mortar. Dried powder 100 mg was extracted with 70 % methanol 1 mL. Extract solution was centrifuged at 1.0×10^4 g and supernatant was tested.

Analyses of secondary metabolites

DPPH radical scavenging activity was measured according to the method by Brand-williams, Cuvelier, and Berset (1995). The supernatant 100 μ L was added to 6×10^{-5} M DPPH MeOH solution and incubated 30 min. Absorbance was measured at 517 nm with a spectrophotometer (Photolab 6100vis, WTW, Germany). Antioxidant activity was expressed as ascorbic acid equivalent.

TPC were analyzed with Folin-Ciocalteu colorimetric methods (Ainsworth and Gillespie, 2007). The supernatant 50 μ L was collected to 2 mL micro tube, and 10% Folin-Ciocalteu solution 750 μ L and distilled water 135 μ L was added and vortexed. 600 μ L 700mM Na_2CO_3 was added and incubated 2 h with room temperature. Absorbance was measured at 765 nm. The result was expressed as gallic acid equivalent per dry weight.

TFC were determined by using aluminum chloride colorimetric (Dewanto et al., 2002). The supernatant 150 μ L was collected 2mL micro tube and was mixed with 135 μ L distilled water and 45 μ L NaNO_2 . After 5 min, 150 μ L 10% AlCl_3 aqueous solution 90 μ L was added. After another 5 min, 1 M NaOH aqueous solution 300 μ L and distilled water 165 μ L was added and incubated for 6 min. Absorbance

was measured at 510 nm. The result was expressed as catechin equivalent per dry weight.

Statistical analysis

The experiment was conducted using a randomized complete block design and all measurements were replicated three times. Statistical analysis was performed using SPSS (SPSS Statistics 23, IBM, USA). Means were compared using Duncan's multiple range tests.

RESULTS AND DISCUSSION

Spatial distributions of chlorophyll fluorescence and secondary metabolites

contents

In the same leaves, F_v/F_m values were different according to the time and locations exposed to different UV-B intensities, while no significances were observed in TFC and TPC (Fig. 1). The F_v/F_m values at T5 (5-day stress from DAT 38) decreased with the time elapsed. The F_v/F_m values were lower at the locations where the amount of UV-B light was higher. Since the amount of UV-B lights received by leaves was uneven by location in leaves, the F_v/F_m values showed different distributions. Wargent et al. (2015) and Yan et al. (2016) reported that UV-B stress affects the maximum quantum efficiency of photosystem II, resulting in lower F_v/F_m value.

The dispersion of the secondary metabolites concentration was homogenized. In the same leaves, F_v/F_m values were different according to locations exposed to different UV-B intensities, while no significances were observed in TFC and TPC (Fig. 2). In previous researches, flavonoids were accumulated in roots although flavonoid biosynthesis enzyme synthesis is light-dependent (Buer et al., 2007). UV-B did not efficiently penetrate tissue, but regulated UVR-8 localized in mesophyll to protect plants from damage effects of UV-B. UVR8 signaling mediated different tissues to develop and defend to UV-B (Bernula et al., 2017). Therefore, although the distribution of UV-B stress varied depending on the UV-B light distributions on leaves of matured kales, the secondary metabolites production was evenly distributed.

Also, the secondary metabolite concentration was not proportional to the F_v/F_m value indicating the degree of stress.

Chlorophyll fluorescence

The F_v/F_m values of kales at UV-B stresses decreased gradually (Fig. 3). Compared to the control, the F_v/F_m values immediately dropped below 0.8 when the plants exposed to UV-B radiation. After stopping UV-B irradiation, the F_v/F_m values were slightly recovered but did not reach 0.8. Measuring F_v/F_m value can identify the changes in the properties of photosynthetic mechanism caused by environmental stresses (Fu et al., 2012). The plants with a relatively low F_v/F_m value showed a slow growth because of the decrease in photosynthetic rate (Boese et al., 1997). The longest UV-B stress such as T5 showed a continuously-low value of F_v/F_m compared the control.

Plant growth

UV-B stress treatment had a negative effect on shoot growth of kales (Fig. 4). No significant change between the control and both UV-B short-term treatment periods as a day or two days were observed in fresh weight. The shoot growth from five days UV-B treatment group was inhibited by UV-B. Fresh weight of five days UV-B treatment group was 40% lower than control. These results indicated that UV-B stress treatment induced the decrease in photosynthesis rate (De la rosa et al., 2003; Soheila, 2000). This suggests that even though UV-B stress treatment increases the concentration of secondary metabolites, the total yield can be reduced.

Secondary metabolites concentration

TPC, TFC, and antioxidant capacity significantly increased at T5 compared to the control (Fig. 5). All the secondary metabolites of kale were enhanced at longer UV-B stresses, while TPC decreased rather than the control and then increased again at shorter stresses. However, most of the previous studies did not show such a decrease in TPC. Increase in TPC at UV-B stress has already been reported (Hideg et al., 2013; Lee et al., 2014; Scattiono et al., 2014). According to Du et al. (2014), UV-B stress treatment of broccoli resulted in a decrease in soluble phenolic contents. The decrease in TPC is due to the scavenge of radicals caused by stress treatment (Kalin et al., 2015). TFC increased in proportion to UV-B stress treatment period to to reduce photoinhibition (Morales et al, 2013). Although antioxidant capacity increased gradually, it was not dramatic compared to flavonoids or phenolic concentrations.

Total secondary metabolites amount

Total amount of TPC and TFC significantly decreased at T5 because of the decrease in dry weight (Fig. 6). TPC, and antioxidant capacity at T5 were significantly higher than any other treatments (Fig. 5) but dry weight was the lowest. Considering total amounts of secondary metabolites, T2 was the optimum treatment period, in which the concentration was lower but the dry weight higher than T5. This is because the dry weight had a greater effect on the total amount than the secondary metabolites concentration induced by UV-B radiation.

In the previous studies, moderate drought stress promoted not only secondary metabolites but also growth thereby increased total production (Zhang et al., 2017).

UV-A stress treatment also enhanced both growth and secondary metabolites (Lee et al., 2014). It is easy to determine the stress treatment period when secondary metabolites concentration and growth are simultaneously promoted through stress treatments. However, UV-B stress treatment suppressed the growth of plants (Tsormpatsdis et al., 2010; Brechner et al., 2011). To maximize the production of secondary metabolites in plant factories, the growth of crops well as the concentration of secondary metabolites are also important. Therefore, it is more efficient to use short-term stresses to reduce the losses of fresh and dry weights.

CONCLUSION

From practical aspects, it is adequate to apply UV-B stress near harvest to enhance the secondary metabolites of kales grown in plant factories. Differences in secondary metabolites accumulation at uneven UV-B distributions in leaves at harvest stage were analyzed using spatial chlorophyll fluorescence images. Also the optimal UV stress periods before harvest was determined to maximize the total amounts of the secondary metabolites production. Distribution of chlorophyll fluorescence in leaves were different according to the locations exposed to different UV-B intensities, while no significances were observed in total phenolic contents and total flavonoid contents. This indicated that accumulation of the secondary metabolites is not always proportional to the local F_v/F_m values. Total phenolic contents, total flavonoid contents, and antioxidant capacity were significantly higher at 5-day UV-B treatment before harvest. However, 2-day UV-B treatment was optimum considering total amounts of the secondary metabolites. This is because the dry weight had a greater effect on the total amount than its concentration. To maximize the secondary metabolites of kales with UV-B treatment, it would be more efficient to use short-term stresses to minimize the loss of fresh and dry weights.

Tables

Table 1. Minimum to maximum ranges of the UV-B treatments.

UV-B treatment	Control	T5-L	T5-M	T5-H
UV-B range ($\text{W} \cdot \text{m}^{-2}$)	0	0 – 3	3 – 6	6 – 9

Table 2. Starting days of the UV-B treatments before harvest.

UV-B treatment	Control	T1	T2	T3	T4	T5
Starting days before harvest	0	1	2	3	4	5

Figures

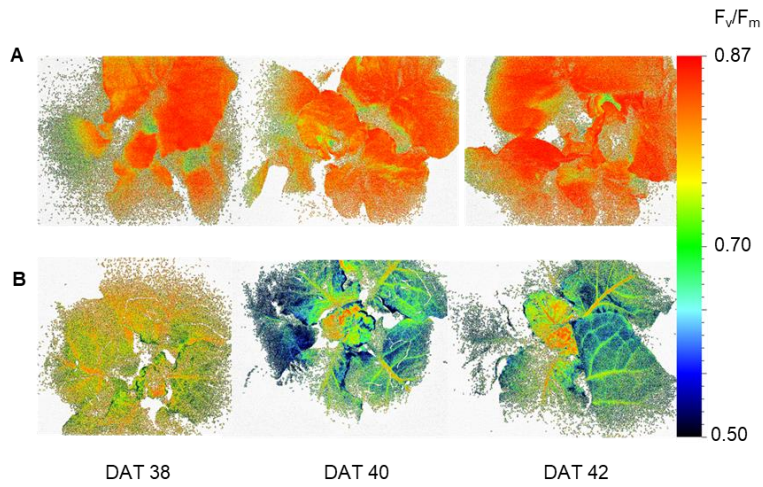


Fig. 1. Changes in spatial images of chlorophyll fluorescence (F_v/F_m) in leaves of kale at 38, 40 and 42 days after transplanting under no UV-B (A) and 4.2 $W \cdot m^{-2}$ UV-B treatment (B) for 4 h a day.

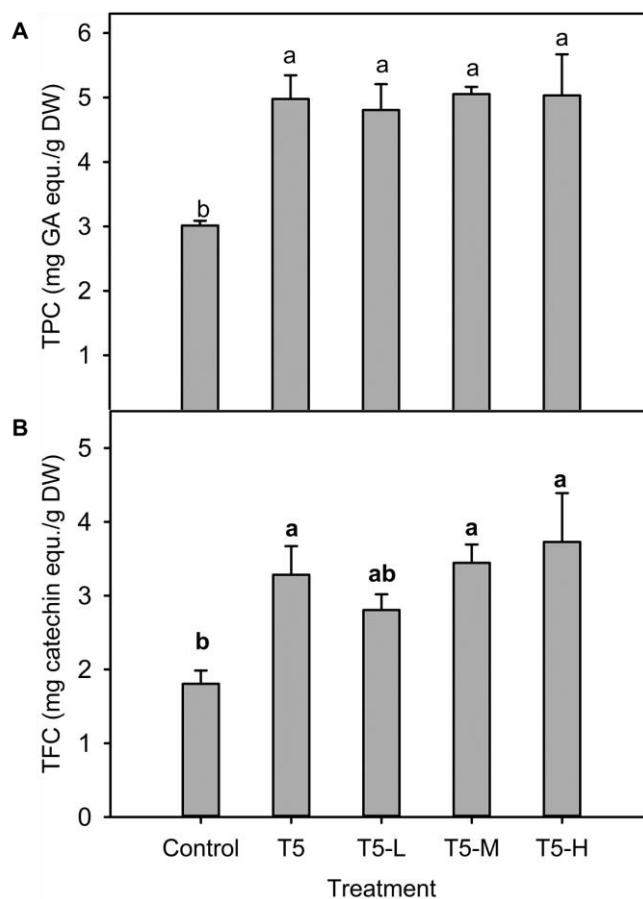


Fig. 2. Total phenolic content (TPC, A) and total flavonoid content (TFC, B) per dry weight by location in leaves exposed at different UV-B intensities. Vertical bars represent the standard error of the mean ($n = 3$). Different letters indicate significant difference ($p < 0.05$) according to Duncan's multiple range test. See Table 1 for the treatments.

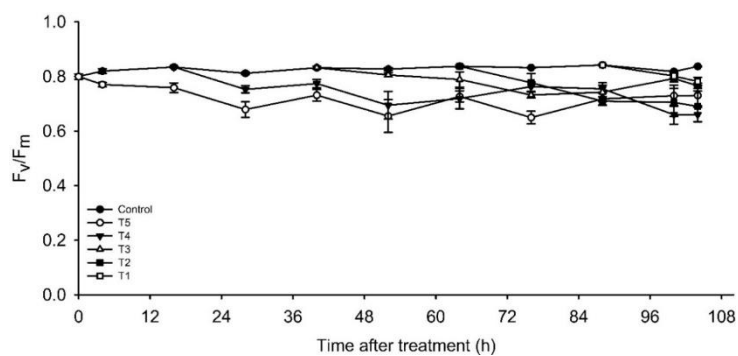


Fig. 3. Changes in chlorophyll fluorescence (F_v/F_m) of kale at different UV-B stress treatments before harvest. Vertical bars represent the standard error of the mean ($n = 3$). See Table 2 for the treatments.

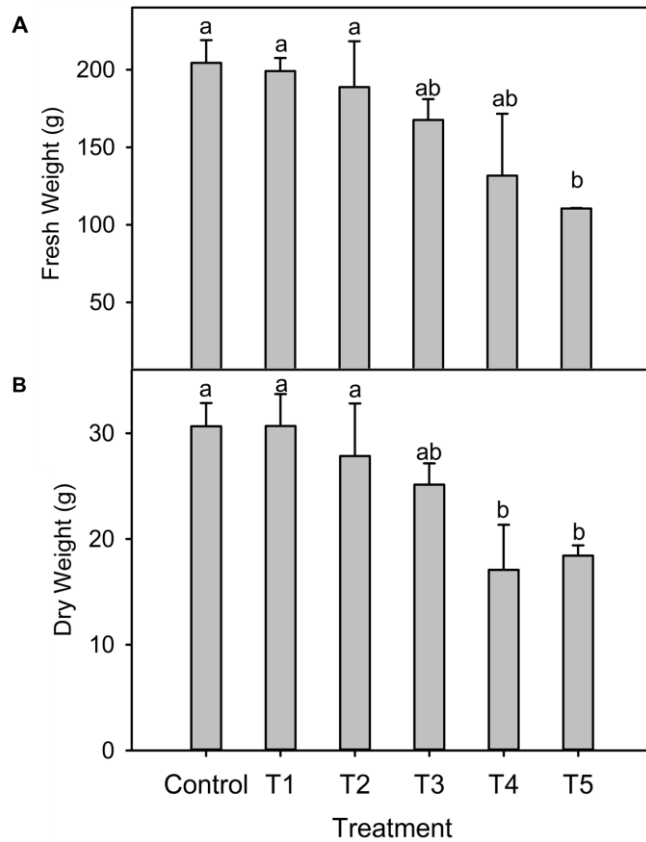


Fig. 4. Shoot fresh (A) and dry (B) weights of kales at different UV-B stress treatments. Vertical bars represent the standard error of the mean ($n = 3$). Different letters indicate significant difference ($p < 0.05$) according to Duncan's multiple range test. See Table 2 for the treatments.

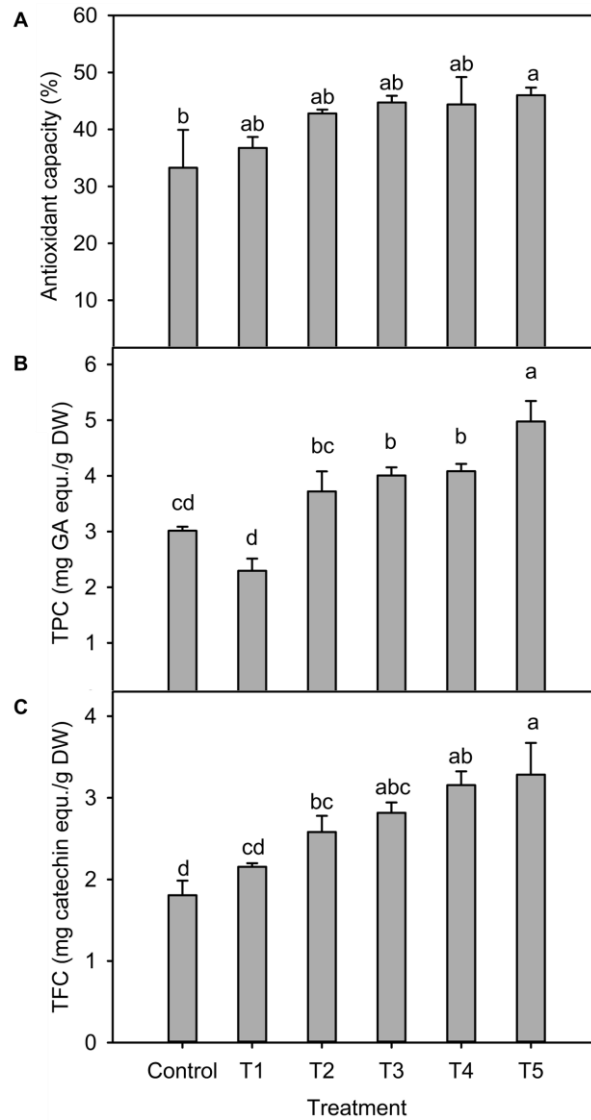


Fig. 5. Antioxidant capacity (A), total phenolic content (TPC, B) and total flavonoid content (TFC, C) per dry weight at different UV-B stress treatments. Vertical bars represent the standard error of the mean ($n = 3$). Different letters indicate significant difference ($p < 0.05$) according to Duncan's multiple range test. See Table 2 for the treatments.

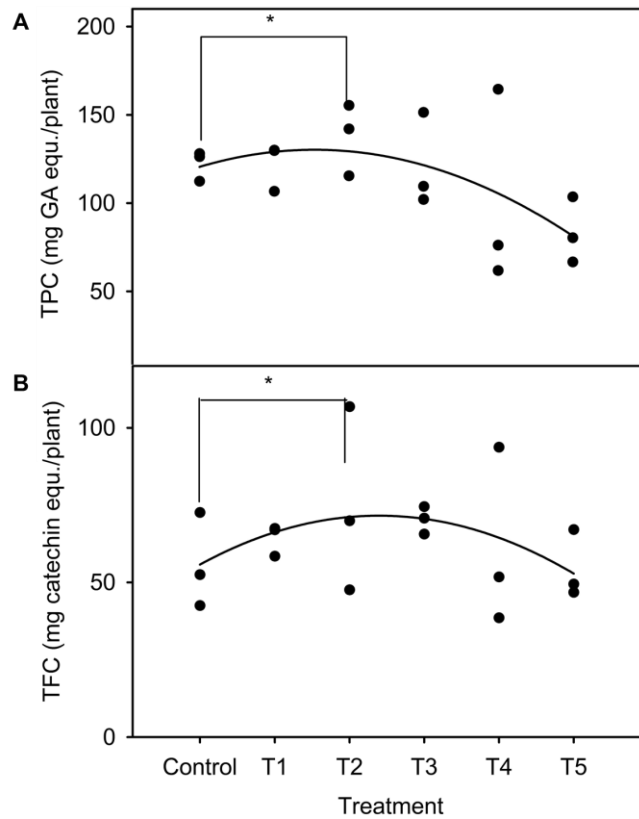


Fig. 6. Total phenolic content (TPC, A) and total flavonoid content (TFC, B) per plant at different UV-B stress treatments. Vertical bars represent the standard error of the mean (n=3). See Table 2 for the treatments. Statistically significant difference is indicated, *P=0.05.

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ABSTRACT IN KOREAN

최근 UV-B 가 식물공장에서 재배되는 케일의 이차대사산물을 향상시키기 위해서 사용되고 있다. 그러나 기존 UV-B 스트레스는 생육 초반에 적용되어 수확기 작물의 이차대사산물 증진에 효과가 있을 지 아직 불분명하다. 본 연구의 목적은 엽록소 형광분포 이미지를 이용하여 수확 단계에서 성숙한 케일 잎의 불균일한 UV-B 분포와 이차대사산물 축적과의 관계를 분석하고, 식물공장에서 재배되는 케일의 이차대사산물 생산의 최대화를 위한 수확 전 UV 스트레스의 최적 처리 시기를 결정하는 것이다. 케일 “만추 콜라드”는 온도 20°C, 상대 습도 70%, 광도 $350 \mu\text{mol m}^{-2}\text{s}^{-1}$, 명기/암기 16 h/8 h 에서 재배하여 정식후 42 일에 수확하였다. 광원은 LED (R : B : W = 8 : 1 : 1)을 사용하였다. 먼저, 엽면의 UV-B 조사량 분포에 따른 엽록소형광(F_v/F_m)을 엽록소 형광이미지 분석기로 측정 하였다. 또한 수확 5, 4, 3, 2, 1 일 전부터 하루 4 시간 동안 4.2 W.m^{-2} UV-B 에 노출시켰다(T5, T4, T3, T2, T1). 수확 후 각 처리 별 생체중, 건물중, 총 페놀릭 함량(TPC), 총 플라보노이드 함량(TFC) 및 항산화능을 비교하였다. 동일한 케일 잎에서도 UV-B 가 조사된 양에 따라 F_v/F_m 값이 상이하였지만, TPC 와 TFC 는 위치별 유의적인 차이가 없었다. 즉, 이차대사산물의 증진이 국부적인 F_v/F_m 에 비례하지 않았다. UV-B 스트레스

처리는 이차대사산물을 증진시키고 생체중과 건조중을 감소시켰다. T5 에서
항산화능, TPC, TFC 는 대조군에 비해 유의적으로 높았지만 건조중은 가장
낮았다. 총 이차대사산물 생산량을 고려할 경우, T2 가 가장 적합한 처리
기간이었다. 실제로 T2 가 T5 에 비해서 농도는 낮았지만 건물중은 높았다. 그
이유는 UV-B 조사에 의한 이차대사산물의 농도가 증진보다 건물중 감소가
이차대사산물 총 생산량에 더 큰 영향을 미쳤기 때문이다. 스트레스 처리를
통해 케일의 이차대사산물 최대화하기 위해선 짧은 기간 스트레스를 처리하는
것이 생체중과 건조중의 감소를 줄여 더 효율적일 것으로 판단된다.

주요어: 엽록소형광이미지, 총페놀릭 함량 총플라보노이드 함량, 항산화능

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